

FINAL REPORT

Test Facility Study No. 511870

Determination of Physico-Chemical Properties of MLA-3202

- Validation of an analytical method
- Water solubility
- Partition coefficient

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1. STATEMENT OF GLP COMPLIANCE

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with:

- OECD Principles of Good Laboratory Practice.
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

Except for the following:

- The characterization of the test item supplied by the sponsor was conducted in a GLP environment.

The data generated and reported are considered to be valid.

Charles River Den Bosch

A handwritten signature in blue ink, appearing to read "M.J.C. Brekelmans". It consists of several slanted strokes forming the letters, with a horizontal line underneath.

Signature:

Name: M.J.C. Brekelmans, MSc.

Title: Study Director

Date:February 27, 2017.....

2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

Study title: Determination of physico-chemical properties of MLA-3202.

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below:

Project 511870

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study	Study Plan	23-May-2016	23-May-2016	23-May-2016
	Study Plan Amendment 01	13-Sep-2016	13-Sep-2016	13-Sep-2016
	Study Plan Amendment 02	08-Dec-2016	08-Dec-2016	08-Dec-2016
	Study Plan Amendment 03	16-Jan-2017	16-Jan-2017	16-Jan-2017
	Study Plan Amendment 04	16-Jan-2017	16-Jan-2017	16-Jan-2017
	Report	31-Jan-2017	02-Feb-2017	02-Feb-2017
Process	Analytical and physical chemistry	06-Jun-2016	21-Jun-2016	23-Jun-2016
	Test Item Handling			
	Exposure			
	Observations/Measurements			
	Specimen Handling			
Test Item Receipt		22-Aug-2016	02-Sep-2016	09-Sep-2016
	Test Item Handling			
Analytical and physical chemistry		05-Sep-2016	22-Sep-2016	30-Sep-2016
	Test Item Handling			
	Exposure			
	Observations/Measurements			
	Specimen Handling			
Test Item Receipt		21-Nov-2016	21-Nov-2016	21-Nov-2016
	Test Item Handling			

Analytical and physical chemistry	01-Dec-2016	28-Dec-2016	28-Dec-2016
Test Item Handling			
Exposure			
Observations/Measurements			
Specimen Handling			

*TFM=Test Facility Management SD = Study Director

The facility inspection program is conducted in accordance with Standard Operating Procedure.

The review of the final report was completed on the date of signing this QA statement.

Charles River Den Bosch

Signature:*Ria van 't Klooster*.....

Name:

**Ria van 't Klooster, BSc
Quality Assurance Auditor**

Date:*27-feb-2017*.....

3. SUMMARY

The results of the physico-chemical properties of the test item are given below.

Parameter	Guideline(s)	Result	Comment
Analytical method	SANCO 3029	validated	
Water solubility	EC A.6 OECD 105 OPPTS 830.7840	0.54 mg/L	at 20°C by the column elution method
Partition coefficient	EC A.8 OECD 117 OPPTS 830.7570	Multiple peaks with $\log P_{ow} \geq 5.3$	HPLC method

4. INTRODUCTION

4.1. Study schedule

Experimental starting date 14 June 2016
Experimental completion date 08 December 2016

4.2. Purpose of the study

The purpose of the study was to determine the following physico-chemical properties for MLA-3202:

- Validation of an analytical method
- Water solubility
- Partition coefficient

4.3. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

4.4. Responsible personnel

4.4.1. Test facility

Study Director M.J.C. Brekelmans, MSc.

4.4.2. Sponsor Representative

Study Monitor Audrey Batoon, Ph.D.

5. MATERIALS

5.1. Test item

5.1.1. Test item information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

5.1.2. Study specific test item information

Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

5.2. Electronic systems for data acquisition

System control, data acquisition and data processing were performed using the following programs:

- Empower 3 database version 7.21 (Waters, Milford, MA, USA)
- MassLynx version 4.1 (Waters, Milford, MA, USA)

Temperature, relative humidity and/or atmospheric pressure during sample storage and/or performance of the studies was monitored continuously using the following program:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA)

5.3. List of deviations

5.3.1. List of study plan deviations

There were no deviations from the study plan.

5.3.2. List of standard operating procedures deviations

There were no deviations from standard operating procedures (SOPs).

6. VALIDATION OF AN ANALYTICAL METHOD

6.1. Guideline

The study was based on the following guideline:

- European Commission: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, section 4) and Annex III (Part A, section 5) of Directive 91/414, SANCO/3029/99 rev. 4 (11/07/00).

6.2. Reagents

Water Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)

Methanol Biosolve, Valkenswaard, The Netherlands

Ammonium acetate Biosolve

All reagents were of analytical grade, unless specified otherwise.

6.3. Performance of the study

An ultra performance liquid chromatographic method with tandem mass spectrometric detection (UPLC-MS/MS) for the quantitative analysis of the test item in water was developed.

Research performed on method development included, but was not limited to, the selection of a solvent to dissolve the test item, a suitable detector and an appropriate combination of column (e.g. stationary phase, dimensions) and mobile phase. Optimizations were performed to obtain a specific method, enhance retention-time stability, limit carry-over and improve the peak shape within the selected calibration range.

Method development is archived in the raw data, only the conditions of the final validated analytical method are issued in the report. The conditions of the validated method were approved in the raw data.

Validation of the analytical method was performed for the following parameters:

Specificity

A test item solution and blank sample were analysed by single injection. The analytical method was found to be specific if the blank chromatogram showed no response for the test item or a response of < 30% of the limit of quantification.

Calibration curve

Calibration solutions were analysed in duplicate. The response of the calibration solutions was correlated with concentration using regression analysis with a 1/concentration² weighting factor. A calibration curve with a coefficient of correlation (*r*) of > 0.99 and back calculated accuracies of the calibration solutions in the range 85-115% was accepted.

Accuracy and repeatability

Quality control (QC) samples were analysed by single injection into the analytical system. The analytical method was considered applicable for the determination of the test item if the mean accuracy was in the range 70-110% and the coefficient of variation was ≤ 20%.

Limit of quantification

The limit of quantification (LOQ) is defined as the lowest concentration level at which an accuracy in the range 70-110% and a repeatability of $\leq 20\%$ is demonstrated. The LOQ was obtained from the data of the accuracy- and repeatability test.

Stability of the analytical system and end solutions

Calibration solutions were injected throughout the validation sequence including the beginning and end. The analytical system and/or end solutions were found to be stable if the coefficient of variation on the responses of the solutions was $\leq 20\%$.

Stability of stock solutions

Stock solutions of the test item were stored at room temperature for at least 12 hours. Additional calibration solutions were prepared and analysed by single injection. The stock solutions were found to be stable if the coefficient of variation on the response factors of the calibration solutions prepared with fresh and stored stock solutions was $\leq 10\%$.

Storage stability of samples

Additional QC samples were prepared and stored in the freezer ($\leq -15^{\circ}\text{C}$) for at least 16 hours. On the day of analysis, the samples were defrosted at room temperature and treated identically as freshly analysed accuracy samples. The stored samples were found to be stable if the mean accuracy was in the range 70-110%.

6.4. Analytical method**6.4.1. Analytical conditions**

Instrument	Acquity UPLC system (Waters, Milford, MA, USA)
Detector	Xevo TQ-S mass spectrometer (Waters)
Column	Acquity UPLC HSS Cyano, 100 mm \times 2.1 mm i.d., $d_p = 1.8 \mu\text{m}$ (Waters)
Column temperature	$40^{\circ}\text{C} \pm 1^{\circ}\text{C}$
Injection volume	5 μL
Mobile phase	10 mM Ammonium acetate in 70/30 (v/v) methanol/water
Flow	0.4 mL/min
MS detection	
Ionisation source	ESI ⁺
Cone voltage	50 V
Acquisition	m/z 398.2 \rightarrow m/z 134 (Collision energy 18 eV) m/z 372.2 \rightarrow m/z 134 (Collision energy 16 eV) m/z 396.2 \rightarrow m/z 134 (Collision energy 16 eV) m/z 400.3 \rightarrow m/z 134 (Collision energy 18 eV)
Quantitation	m/z 398.2 \rightarrow m/z 134

6.4.2. Preparation of solutions

Stock and spiking solutions

Stock solutions of the test item were prepared in methanol at a concentration of 2000 mg/L.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was methanol.

Calibration solutions

Five solutions with the test item in the concentration range of 200 - 30000 µg/L were prepared in methanol from two stock solutions. The solutions were diluted by a factor of 100 with 75/25 (v/v) methanol/water to obtain calibration solutions in the concentration range of 2 -300 µg/L.

Quality control (QC) samples

1 mL water was spiked with the test item at a target concentration of 0.01 or 10 mg/L. The QC samples were diluted in a 1:3 (v:v) ratio with methanol and analysed. If necessary, the samples were further diluted with 75/25 (v/v) methanol/water to obtain concentrations within the calibration range.

The blank QC sample consisting of blank medium was treated similarly to the QC samples.

6.5. Formulas

Response (R) Peak area of the test item [units]

$$\text{Response factor (R}_f\text{)} \quad R_f = \frac{R}{C_N}$$

where:

C_N = nominal concentration [$\mu\text{g/L}$]

Calibration curve

$$R = a C_N + b$$

where:

a = slope [units \times L/ μ g]

b = intercept [units]

Analysed concentration (C_A)
$$C_A = \frac{(R - b)}{a} \times d \quad [\mu\text{g/L}]$$

where:

$d \equiv$ dilution factor

Accuracy $\frac{C_A}{C_N} \times 100$ [%]

6.6. Results

6.6.1. Specificity

Chromatograms of a QC sample at the LOQ level and blank QC sample are shown in [Figure 1](#) and [Figure 2](#), respectively.

The chromatogram of the QC sample showed one test item peak for the m/z 398.2 → m/z 134 transition. The area of this peak was used as response in the calculations.

The chromatogram of the blank accuracy sample showed no peak at the retention time of the test item. Since no interferences were detected, the specificity requirements were met and the analytical method was found to be specific for the test item.

6.6.2. Calibration curve

The calibration line was constructed using all data points. [Figure 3](#) illustrates the calibration curve and [Table 1](#) shows the statistical parameters. There was a linear relationship between response and test item concentration in the range of 2 – 300 µg/L (in end solution). Since the coefficient of correlation (r) was > 0.99 and the back calculated accuracies of the data points were in the range 85–115% the calibration line was accepted.

Table 1
Statistical parameters of the calibration curve

Slope	2.44×10^4
Intercept	-1.20×10^2
Weighting factor	1/concentration ²
r	0.9997

6.6.3. Accuracy and repeatability

The results of the QC samples are given in [Table 2](#). Since the mean accuracy at each concentration level fell in the criterion 70–110% and the coefficient of variation was $\leq 20\%$ the analytical method was accepted for the analysis of the test item in water in the target concentration range of 0.01 - 10 mg/L.

Table 2
Accuracy and repeatability

Target	Concentration [mg/L]		Accuracy [%]		Coefficient of variation [%]
	Nominal	Analysed	Individual	Mean	
0.01	0.0100	0.0112	112	102	5.3
	0.0100	0.00991	99		
	0.0100	0.0100	100		
	0.0100	0.0102	102		
	0.0100	0.00990	99		
10	10.0	9.70	97	98	0.85
	10.0	9.74	97		
	10.0	9.91	99		
	10.0	9.84	98		
	10.0	9.80	98		

6.6.4. Limit of quantification

The limit of quantification (LOQ) was assessed at 0.01 mg/L in water.

6.6.5. Stability of the analytical system and end solutions

The results of the stability of the analytical system and end solutions are given in [Table 3](#). Since the coefficient of variation at both concentration levels was $\leq 20\%$ the analytical system and end solutions were stable over at least a 2.31 hour time interval.

Table 3
Stability of the analytical system and end solutions

Nominal concentration [$\mu\text{g}/\text{L}$]	Elapsed time [hours]	Coefficient of variation [%]
2	2.59	4.2 (n=8)
300	2.31	0.91 (n=8)

6.6.6. Stability of stock solutions

Stability of the stock solutions was determined in project 514869. The coefficient of variation on the response factors of the calibration solutions prepared with fresh and stored stock solutions was 1.2%. Since the value was $\leq 10\%$ the stock solutions were stable when stored at room temperature for at least 1 day.

6.6.7. Storage stability of samples

The results of the storage stability of the test item in the freezer are given in [Table 4](#). Since the mean accuracy of the frozen QC samples fell in the criterion 70-110% the samples were stable when stored in the freezer.

Table 4
Storage stability of samples

Target	Concentration [mg/L]		Accuracy [%]	
	Nominal	Analysed	Individual	Mean
0.01	0.0100	0.00987	99	99
	0.0100	0.00983	98	
10	10.0	9.57	96	97
	10.0	9.79	98	

6.7. Conclusion

The analytical method was validated for the following parameters:

Specificity	specific
Calibration curve	r = 0.9997
Accuracy	102 and 98%
Repeatability	5.3 and 0.85%
Limit of quantification	0.01 mg/L
Stability analytical system and end solutions	stable
Stability stock solutions	stable
Storage stability of samples	stable

6.8. Figures

Note: the intensity scales are different in Figure 1 and Figure 2.

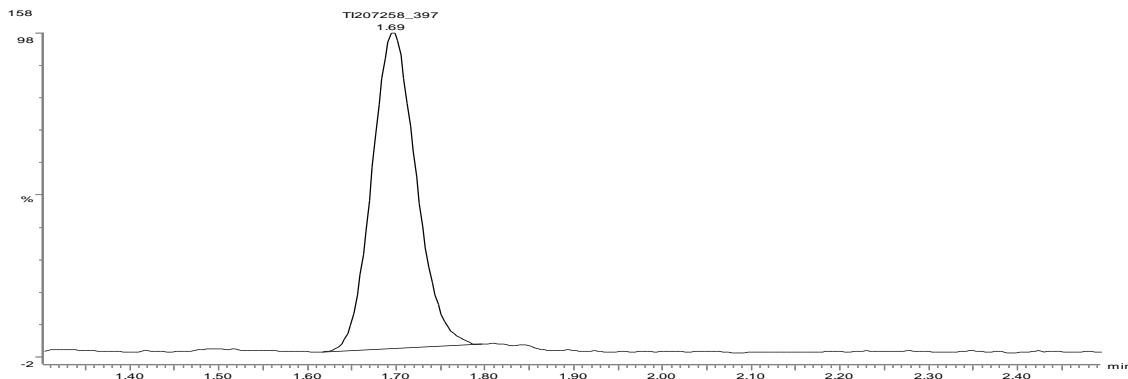


Figure 1
UPLC-MS/MS chromatogram of a 0.01 mg/L QC sample [res. id. 158]

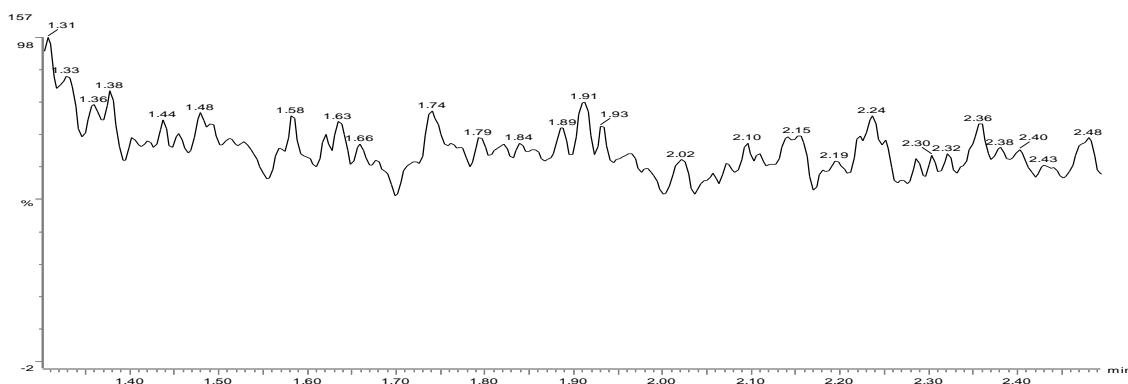


Figure 2
UPLC-MS/MS chromatogram of the blank QC sample [res. id. 157]

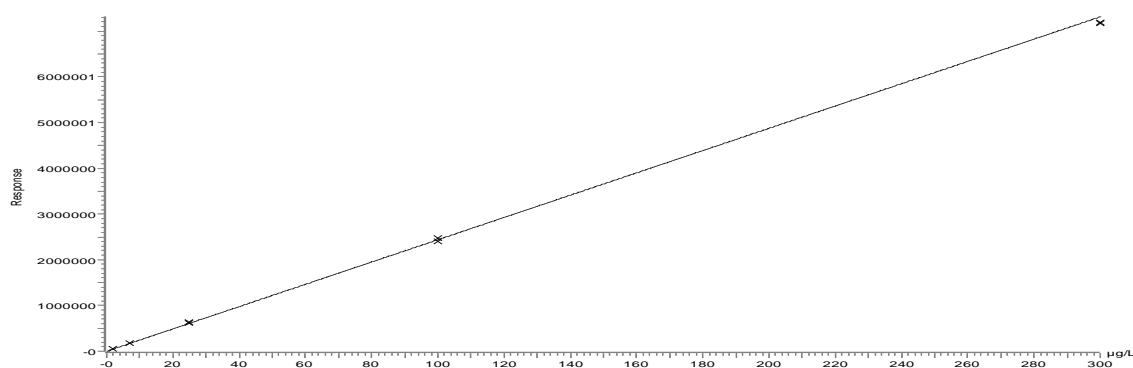


Figure 3
Regression line: response of the test item as a function of concentration

7. WATER SOLUBILITY

7.1. Guidelines

- European Community (EC), EC no. 260/2014, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.6: "Water Solubility", Official Journal of the European Union no. L81, March 19, 2014.
- Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 105: "Water Solubility", July 27, 1995.
- United States Environmental Protection Agency (EPA), Product Properties Test Guidelines no. OPPTS 830.7840: "Water Solubility: Column Elution Method; Shake Flask Method", March 1998.

7.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Double distilled water	Waldeck, Münster-Roxel, Germany
Methanol	Biosolve. Valkenswaard, The Netherlands
Ammonium acetate	Biosolve
Acetone	VWR International, Leuven, Belgium
Sodium Chloride	Merck, Darmstadt, Germany
Sodium azide	Merck

All reagents were of analytical grade, unless specified otherwise.

7.3. Performance of the study

Two methods are available for the determination of the water solubility of a solid test item:

Flask method

The mass concentration of the test item in aqueous solutions saturated with the test item will be determined after stirring or shaking at a specific temperature for a specific period of time. The method can be used for test items with a water solubility of $\geq 10^{-2}$ g/L.

Column elution method

The method is based on the elution of the test item with water from a column which is charged with an inert support material, such as bare silica particles, coated with an excess of the test item. It can be applied to test items with a water solubility of $< 10^{-2}$ g/L.

A preliminary flask water solubility test was performed by adding 10 μ L test item to 100 mL double distilled water, with slow stirring at 100 rpm for at least 24 hours.

Solubilities measured however were $< 10^{-2}$ g/L. Therefore, the column elution method was considered to be a good alternative for performance of a main study.

7.3.1. Main study

A solution with an absolute test item quantity of 49.2 mg was prepared in acetone. The solution was mixed with 5 g LiChroprep Si 60 25-40 µm column material (Merck, Darmstadt, Germany). Acetone was completely evaporated at 40°C using a rotary evaporator. The loaded carrier material was stored overnight at room temperature.

A 150 mm × 4.6 mm i.d. column was filled with the carrier material and 0.5 µm frits were used to enclose the column. Double distilled water was pumped through the column until it was completely filled. The system was allowed to equilibrate for two hours.

After the equilibration period, the column was eluted with double distilled water at a flow rate of 24 mL/hour. Ten consecutive samples of 2 mL were taken. The flow was halved to 12 mL/hour and ten consecutive samples of 2 mL were taken.

Analysis was performed on subsamples of 0.25 mL. The samples were diluted in a 1:3 (v:v) ratio with methanol and analysed.

The pH was measured from the water fractions that were collected for the determination of the flow rates. After sampling, the remaining parts of the water samples were combined for pH measurement. Each fraction was diluted in a 9:1 (v:v) ratio with 0.1 M sodium chloride in order to obtain a high electrolyte concentration for accurate pH measurement. The sodium chloride solution contained 0.0009% (w/v) sodium azide.

The eluates were checked for the presence of undissolved particles using a turbidimeter.

The experiment was also performed using a column filled with blank carrier material. The procedure was identical to the procedure used for the column loaded with test item. At each flow rate, two consecutive samples were taken for analyses.

The column elution method was performed at $19.9^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$.

7.4. Analytical method

7.4.1. Analytical conditions

Quantitative analysis was performed according to the analytical method as validated in Chapter 6.

Turbidity measurements:

Instrument	2100 AN IS Laboratory Turbidimeter (Hach Company, Loveland, Colorado, USA)
Unit	NTU
Calibration range	< 0.1 to 7500 NTU

7.4.2. Preparation of the calibration solutions

Stock solutions

Stock solutions of the test item were prepared in methanol at a concentration of 2000 mg/L.

Calibration solutions

Calibration solutions in the concentration range of 200 - 30000 µg/L were prepared in methanol from two stock solutions. The solutions were diluted by a factor of 100 with 75/25 (v/v) methanol/water to obtain calibration solutions in the concentration range of 2 - 300 µg/L.

7.4.3. Sample injections

Calibration solutions were injected in duplicate. Test samples were analysed by single injection.

7.4.4. Calibration curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration² weighting factor. The coefficient of correlation (*r*) was > 0.99 for each curve.

7.5. Interpretation

7.5.1. Specifications

The water solubility of a test item is specified by the saturation mass concentration of the test item in water at a given temperature and is expressed in $\mu\text{g/L}$.

7.5.2. Formulas

Response (R) Peak area of the test item [units]

$$\frac{\text{highest value} - \text{lowest value}}{\text{mean value}} \times 100\%$$

where:

'mean value' is the mean of the highest and lowest value.

Calibration curve

$$R = aC_N + b$$

where:

C_N = nominal concentration [$\mu\text{g/L}$]

a = slope [units \times L/ μ g]

b = intercept [units]

Analysed concentration (C_A)

$$C_A = \frac{(R - b)}{a} \times d \quad [\mu\text{g/L}]$$

where:

d = dilution factor

7.6. Results

7.6.1. Preliminary test

It was observed that the test item formed a jelly-like substance, floating freely in the bulk of the water phase. It was not possible to take a sample from the clear water phase without taking also undissolved test item. In order to obtain a sample without undissolved test item filtration and centrifugation were tested. Filtration failed since the test item adsorbs to the 0.2 µm FP 30/0.2 CA-S filter used. Centrifugation failed since concentration decreased with the number of centrifugation steps (5 minutes at 12000 rpm, 20°C). Based on this it was concluded that the flask method is not applicable for determination of the water solubility of MLA-3202.

7.6.2. Main study

The results for the samples taken at a flow rate of 12 and 24 mL/h are given in [Table 5](#).

The coefficient of variation (CV) ($n = 10$) was $\leq 30\%$ at both flow rates. It demonstrated that the system was equilibrated at all flow rates.

The maximum difference (MD) on the mean values of the samples at both flow rates was also $\leq 30\%$. Based on this, the water solubility of the test item is given as the mean value of these measurements and in the conclusion rounded to two significant digits.

The turbidity of the eluates was < 0.2 NTU. According to this, no undissolved particles were detected in the eluates.

No test item was detected in the samples from the blank column.

Table 5
Main study - water solubility of the test item

Flow rate [mL/h]	Sample no.	Concentration analysed					pH
		Individual [µg/L]	Mean ¹ [µg/L]	CV [%]	Mean ² [µg/L]	MD [%]	
24	1	514	512	4.2	536	9.1	7.8
	2	501					
	3	522					
	4	485					
	5	476					
	6	501					
	7	516					
	8	534					
	9	521					
	10	548					
12	1	645	560	10			7.7
	2	618					
	3	608					
	4	592					
	5	578					
	6	563					
	7	528					
	8	508					
	9	486					
	10	475					

¹ Mean of the concentrations obtained at one flow rate.

² Mean of the concentrations obtained at both flow rates.

7.7. Conclusion

The column elution method was applied for the determination of the water solubility of MLA-3202.

The water solubility of the test item at 20°C was 0.54 mg/L.

The pH of the aqueous samples was 7.7 – 7.8.

8. PARTITION COEFFICIENT

8.1. Guidelines

- European Community (EC), EC no. 2016/266, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.24: "Partition Coefficient (n-octanol/water), High Performance Liquid Chromatography (HPLC) Method", Official Journal of the European Union no. L54, March 1, 2016.
- Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 117: "Partition Coefficient (n-octanol/water), High Performance Liquid Chromatography (HPLC) Method", April 13, 2004.
- United States Environmental Protection Agency (EPA), Product Properties Test Guidelines no. OPPTS 830.7570: "Partition Coefficient (n-octanol/water), Estimation by Liquid Chromatography", August 1996.

8.2. Reagents

Water Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)

Methanol Biosolve, Valkenswaard, The Netherlands

N,N-Dimethylformamide (DMF) Merck, Darmstadt, Germany

All reagents were of analytical grade, unless specified otherwise.

8.3. Performance of the study

Four methods are available for the determination of the partition coefficient (P_{ow}) of the test item. The principle of each method is described below:

Shake flask method

Test item is partitioned in a two-phase system of n-octanol and water. After phase separation, the concentration of the test item in both phases is determined by a suitable analytical method. The shake flask method can be used for test item with a $\log P_{ow}$ value in the range of -2 to 4.

HPLC method

A solution of the test item is injected onto a reversed-phase UPLC column. The $\log P_{ow}$ value is calculated from the retention time of the test item. The HPLC method covers $\log P_{ow}$ values in the range of 0 to at least 6.

Estimation method

The solubility of the test item in n-octanol and in water is determined. The quotient of the n-octanol solubility and the water solubility is an estimation of the P_{ow} . The estimation method can be applied if the shake flask method and HPLC method are not applicable.

Calculation method

The calculation method is based on the theoretical fragmentation of the molecule into suitable substructures for which reliable $\log P_{ow}$ increments are known. The $\log P_{ow}$ is obtained by summing the fragment values and the correction terms for intramolecular interactions. Lists of fragment constants and correction terms are available.

MLA-3202 is a UVCB and contains a number of Amides, tallow, N,N-bis(2-hydropropyl). For a test item that contains several compounds, the HPLC method is the preferred method. Therefore this method was used.

Solutions of reference substances and the test item were analysed. The capacity factor (k') of each compound was calculated from its retention time. The log k' values of the references substances were plotted against the known log P_{ow} values. A linear regression program was used to calculate the calibration curve. Linear regression analysis was performed using the least squares method. The coefficient of correlation (r) was calculated. The log P_{ow} value for the test item was calculated by substituting its mean log k' in the calibration curve. The value of log P_{ow} obtained from duplicate measurements was within ± 0.1 log units.

8.4. Analytical method

8.4.1. Analytical conditions

Instrument	Acquity UPLC system (Waters, Milford, MA, USA)
Detector	Acquity UPLC TUV detector (Waters)
Column	Acquity UPLC HSS T3, 100 mm \times 2.1 mm i.d., $d_p = 1.8 \mu\text{m}$ (Waters)
Column temperature	35°C $\pm 1^\circ\text{C}$
Mobile phase	A - methanol B - water

Gradient¹

Time [minutes]	%A	%B
0	75	25
30	75	25
30.1	100	0
40	100	0
40.1	75	25
60	75	25

Flow 0.4 mL/min

Injection volume 5 μL

UV detection 210 nm

8.4.2. Preparation of the solutions

Solution of the unretained compound

A 5.0 g/L stock solution of formamide (99.2%, [75-12-7], Alfa Aesar, Karlsruhe, Germany) in methanol was used. The stock solution was diluted to obtain an end solution of 75/25 (v/v) methanol/water.

The formamide blank solution was 75/25 (v/v) methanol/water.

¹ A gradient was applied in order to elute components with log Pow > 7.2 from the column. The reference compounds are eluted in the isocratic part of the method and the gradient step was not applied for these injections.

Reference substance solutions

Stock solutions of the reference substances at concentrations of approximately 1 g/L in methanol were used. The stock solutions were diluted to obtain an end solution of 75/25 (v/v) methanol/water.

The blank solution for the mixture of reference substances was 75/25 (v/v) methanol/water.

Reference substance	Purity	CAS number	Supplier	$\log P_{ow}^{\#}$
1,4-Dichlorobenzene	99.9%	106-46-7	Sigma-Aldrich	3.4
Biphenyl	99.8%	92-52-4	Acros Organics	4.0
1,2,4-Trichlorobenzene	99.8%	120-82-1	Acros Organics	4.2
Dibenzyl	99.7%	103-29-7	Acros Organics	4.8
Triphenylamine	99.9%	603-34-9	Acros Organics	5.7
4,4'-DDT	98.7%	50-29-3	Sigma-Aldrich	6.5

values according to the OECD 117 guideline

Acros Organics, Geel, Belgium

Sigma-Aldrich, Steinheim, Germany

Reference substance solution with $\log P_{ow} > 6.5$

A stock solution of benzo[ghi]perylene at a concentration of approximately 1 g/L in DMF was used. The stock solution was diluted to obtain an end solution of 75/25 (v/v) DMF/water.

The blank solution for the mixture of reference substances was 75/25 (v/v) DMF/water.

Reference substance	Purity	CAS number	Supplier	$\log P_{ow}^{\#}$
Benzo[ghi]perylene	97.9%	191-24-2	Sigma-Aldrich	7.2

value according Verschueren, K. (1996) Handbook of Environmental Data on Organic Chemicals, 3rd ed.
Sigma-Aldrich, Steinheim, Germany

Test solution

A 2000 mg/L stock solution of the test item was prepared in methanol. The stock solution was diluted in methanol to a final concentration of 1000 mg/L.

The test item blank solution was methanol.

8.4.3. Injections

The reference substance and test item solutions were injected in duplicate. Blank solutions were analysed by single injection.

8.5. Interpretation

$$\text{Capacity factor (}k'\text{)} = \frac{(t_r - t_0)}{t_0}$$

where:

t_r = retention time

t_0 = mean column dead time

Calibration curve

$$\log k' = a \log P_{ow} + b$$

where:

a = slope

b = intercept

8.6. Results

8.6.1. HPLC Method

UPLC-UV chromatograms of the test item solution and corresponding blank are shown in Figure 4

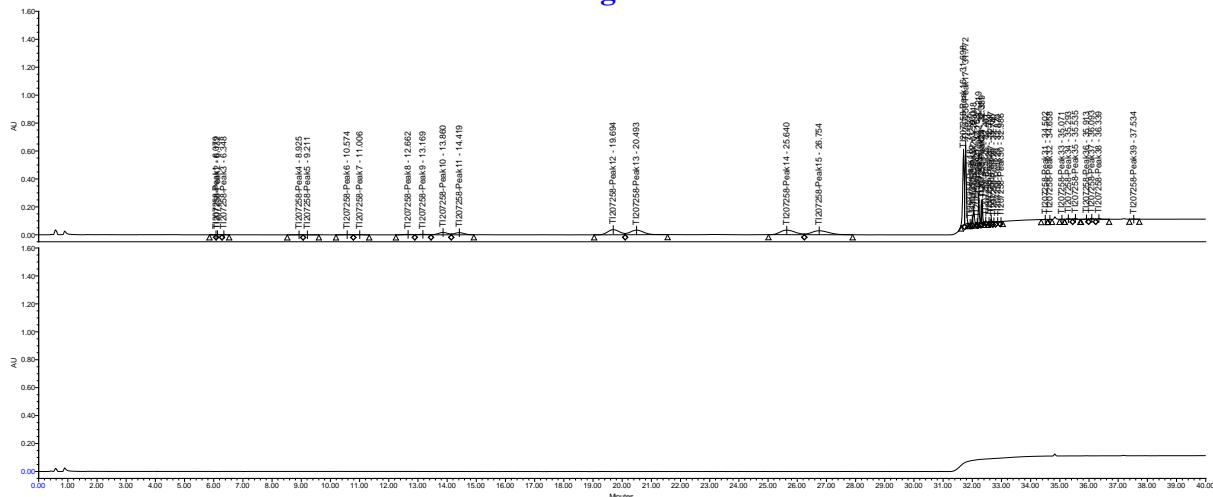


Figure 4. Figure 5 to Figure 8 show enlargements of the test item chromatogram including enlargements of the corresponding blank.

In the chromatogram of the test solution, several peaks were observed.

The results of the HPLC method are given in [Table 6](#). [Figure 9](#) shows the calibration curve of the log k' of the reference substances as function of log P_{ow} . The equation of the regression line was: $\log k' = 0.297 \times \log P_{ow} - 0.608$ ($r = 0.998$, $n = 14$).

Table 6
P_{ow} of the test item

Substance	t _{r,1} [min]	t _{r,2} [min]	mean t _r (n=2)	log P _{ow}	P _{ow}	Area %
Formamide (t ₀)	0.595	0.591	0.593			
1,4-Dichlorobenzene	2.050	2.021		3.4		
Biphenyl	2.779	2.741		4.0		
1,2,4-Trichlorobenzene	3.205	3.160		4.2		
Dibenzyl	4.684	4.630		4.8		
Triphenylamine	8.663	8.649		5.7		
4,4'-DDT	12.542	12.639		6.5		
Benzo[ghi]perylene	19.411	19.925		7.2		
TI207258-Peak1	6.079	6.051	6.065	5.3	2.0 × 10 ⁵	0.052
TI207258-Peak2	6.132	6.143	6.138	5.3	2.1 × 10 ⁵	0.094
TI207258-Peak3	6.348	6.332	6.340	5.4	2.3 × 10 ⁵	0.14
TI207258-Peak4	8.925	8.940	8.933	5.9	8.2 × 10 ⁵	0.11
TI207258-Peak5	9.211	9.273	9.242	6.0	9.3 × 10 ⁵	0.10
TI207258-Peak6	10.574	10.612	10.593	6.2	1.5 × 10 ⁶	0.33
TI207258-Peak7	11.006	11.065	11.036	6.2	1.8 × 10 ⁶	0.29
TI207258-Peak8	12.662	12.716	12.689	6.5	2.9 × 10 ⁶	0.69
TI207258-Peak9	13.169	13.230	13.200	6.5	3.3 × 10 ⁶	0.77
TI207258-Peak10	13.860	13.931	13.896	6.6	4.0 × 10 ⁶	3.7
TI207258-Peak11	14.419	14.488	14.454	6.7	4.6 × 10 ⁶	3.4
TI207258-Peak12	19.694	19.736	19.715	7.1	1.3 × 10 ⁷	9.7
TI207258-Peak13	20.493	20.532	20.513	> 7.2	> 1.6 × 10 ⁷	9.9
TI207258-Peak14	25.640	25.642	25.641	> 7.2	> 1.6 × 10 ⁷	11
TI207258-Peak15	26.754	26.758	26.756	> 7.2	> 1.6 × 10 ⁷	11
TI207258-Peak16	31.698	31.694	31.696	> 7.2	> 1.6 × 10 ⁷	17
TI207258-Peak17	31.772	31.768	31.770	> 7.2	> 1.6 × 10 ⁷	17
TI207258-Peak18	31.927	31.925	31.926	> 7.2	> 1.6 × 10 ⁷	0.016
TI207258-Peak19	32.000	31.997	31.999	> 7.2	> 1.6 × 10 ⁷	0.55
TI207258-Peak20	32.048	32.044	32.046	> 7.2	> 1.6 × 10 ⁷	2.6
TI207258-Peak21	32.168	32.161	32.165	> 7.2	> 1.6 × 10 ⁷	0.10
TI207258-Peak22	32.219	32.216	32.218	> 7.2	> 1.6 × 10 ⁷	3.7
TI207258-Peak23	32.311	32.307	32.309	> 7.2	> 1.6 × 10 ⁷	2.0
TI207258-Peak24	32.339	32.336	32.338	> 7.2	> 1.6 × 10 ⁷	2.6
TI207258-Peak25	32.461	32.457	32.459	> 7.2	> 1.6 × 10 ⁷	0.42
TI207258-Peak26	32.574	32.570	32.572	> 7.2	> 1.6 × 10 ⁷	0.12
TI207258-Peak27	32.647	32.643	32.645	> 7.2	> 1.6 × 10 ⁷	0.52
TI207258-Peak28	32.740	32.736	32.738	> 7.2	> 1.6 × 10 ⁷	0.053
TI207258-Peak29	32.870	32.865	32.868	> 7.2	> 1.6 × 10 ⁷	0.079
TI207258-Peak30	32.986	32.981	32.984	> 7.2	> 1.6 × 10 ⁷	0.025
TI207258-Peak31	34.502	34.496	34.499	> 7.2	> 1.6 × 10 ⁷	0.15
TI207258-Peak32	34.658	34.651	34.655	> 7.2	> 1.6 × 10 ⁷	0.066
TI207258-Peak33	35.071	35.066	35.069	> 7.2	> 1.6 × 10 ⁷	0.066
TI207258-Peak34	35.293	35.287	35.290	> 7.2	> 1.6 × 10 ⁷	0.42
TI207258-Peak35	35.535	35.528	35.532	> 7.2	> 1.6 × 10 ⁷	0.45
TI207258-Peak36	35.913	35.903	35.908	> 7.2	> 1.6 × 10 ⁷	0.22
TI207258-Peak37	36.093	36.087	36.090	> 7.2	> 1.6 × 10 ⁷	0.54
TI207258-Peak38	36.339	36.332	36.336	> 7.2	> 1.6 × 10 ⁷	0.48
TI207258-Peak39	37.534	37.525	37.530	> 7.2	> 1.6 × 10 ⁷	0.073

8.7. Conclusion

The HPLC method was applied for the determination of the partition coefficient (P_{ow}) of MLA-3202.

The P_{ow} and $\log P_{ow}$ values of the test item at neutral pH were:

	P_{ow}	$\log P_{ow}$	Area %
TI207258-Peak1	2.0×10^5	5.3	0.052
TI207258-Peak2	2.1×10^5	5.3	0.094
TI207258-Peak3	2.3×10^5	5.4	0.14
TI207258-Peak4	8.2×10^5	5.9	0.11
TI207258-Peak5	9.3×10^5	6.0	0.10
TI207258-Peak6	1.5×10^6	6.2	0.33
TI207258-Peak7	1.8×10^6	6.2	0.29
TI207258-Peak8	2.9×10^6	6.5	0.69
TI207258-Peak9	3.3×10^6	6.5	0.77
TI207258-Peak10	4.0×10^6	6.6	3.7
TI207258-Peak11	4.6×10^6	6.7	3.4
TI207258-Peak12	1.3×10^7	7.1	9.7
TI207258-Peak13	$> 1.6 \times 10^7$	> 7.2	9.9
TI207258-Peak14	$> 1.6 \times 10^7$	> 7.2	11
TI207258-Peak15	$> 1.6 \times 10^7$	> 7.2	11
TI207258-Peak16	$> 1.6 \times 10^7$	> 7.2	17
TI207258-Peak17	$> 1.6 \times 10^7$	> 7.2	17
TI207258-Peak18	$> 1.6 \times 10^7$	> 7.2	0.016
TI207258-Peak19	$> 1.6 \times 10^7$	> 7.2	0.55
TI207258-Peak20	$> 1.6 \times 10^7$	> 7.2	2.6
TI207258-Peak21	$> 1.6 \times 10^7$	> 7.2	0.10
TI207258-Peak22	$> 1.6 \times 10^7$	> 7.2	3.7
TI207258-Peak23	$> 1.6 \times 10^7$	> 7.2	2.0
TI207258-Peak24	$> 1.6 \times 10^7$	> 7.2	2.6
TI207258-Peak25	$> 1.6 \times 10^7$	> 7.2	0.42
TI207258-Peak26	$> 1.6 \times 10^7$	> 7.2	0.12
TI207258-Peak27	$> 1.6 \times 10^7$	> 7.2	0.52
TI207258-Peak28	$> 1.6 \times 10^7$	> 7.2	0.053
TI207258-Peak29	$> 1.6 \times 10^7$	> 7.2	0.079
TI207258-Peak30	$> 1.6 \times 10^7$	> 7.2	0.025
TI207258-Peak31	$> 1.6 \times 10^7$	> 7.2	0.15
TI207258-Peak32	$> 1.6 \times 10^7$	> 7.2	0.066
TI207258-Peak33	$> 1.6 \times 10^7$	> 7.2	0.066
TI207258-Peak34	$> 1.6 \times 10^7$	> 7.2	0.42
TI207258-Peak35	$> 1.6 \times 10^7$	> 7.2	0.45
TI207258-Peak36	$> 1.6 \times 10^7$	> 7.2	0.22
TI207258-Peak37	$> 1.6 \times 10^7$	> 7.2	0.54
TI207258-Peak38	$> 1.6 \times 10^7$	> 7.2	0.48
TI207258-Peak39	$> 1.6 \times 10^7$	> 7.2	0.073

8.8. Figures

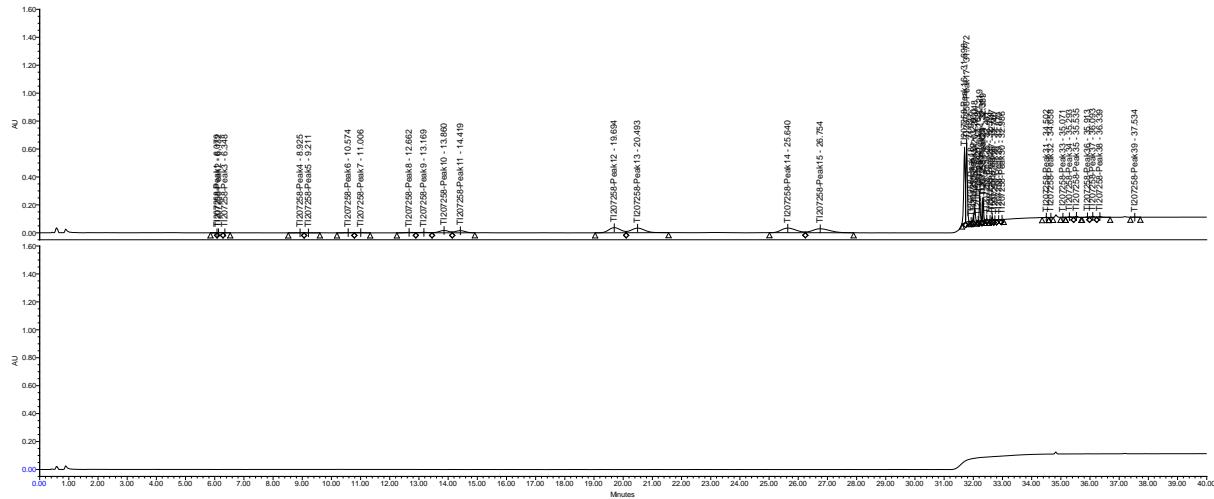


Figure 4
UPLC-UV chromatograms of the 1000 mg/L test solution [top; res. id. 1584] and corresponding blank [bottom; res. id. 1583]

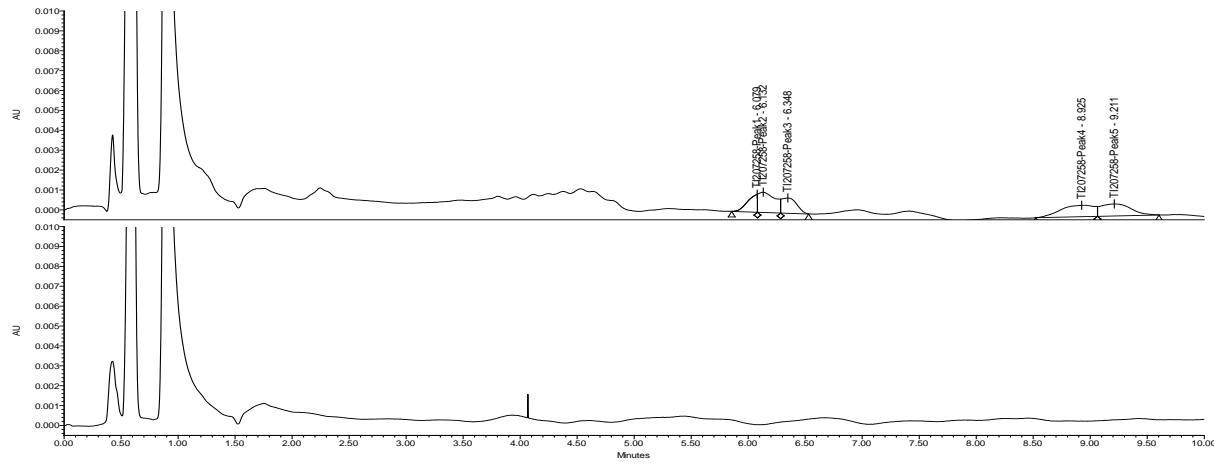


Figure 5
Zoomed UPLC-UV chromatograms of the 1000 mg/L test solution [top; res. id. 1584] and corresponding blank [bottom; res. id. 1583], 0-10 minutes

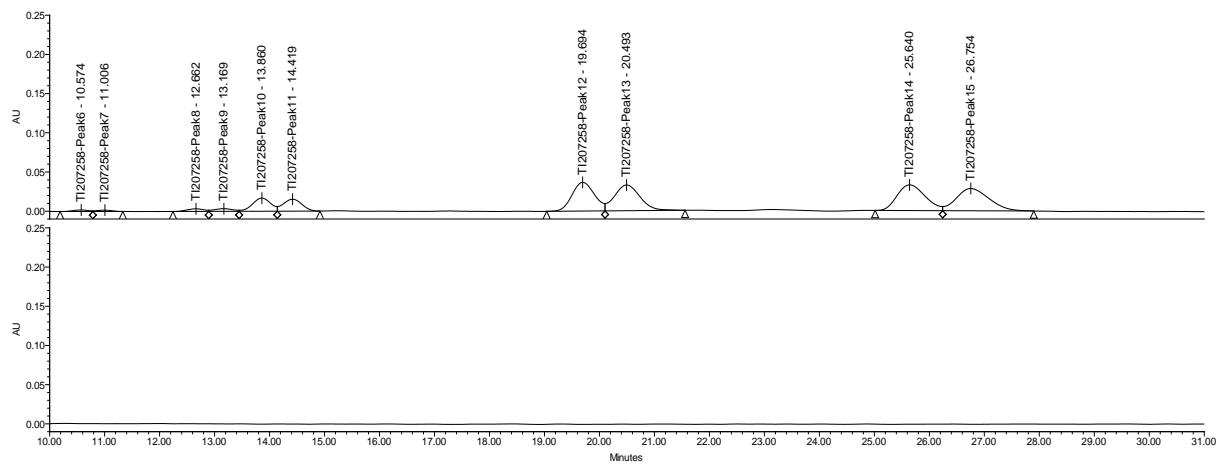


Figure 6
**Zoomed UPLC-UV chromatograms of the 1000 mg/L test solution [top; res. id. 1584]
and corresponding blank [bottom; res. id. 1583], 10-31 minutes**

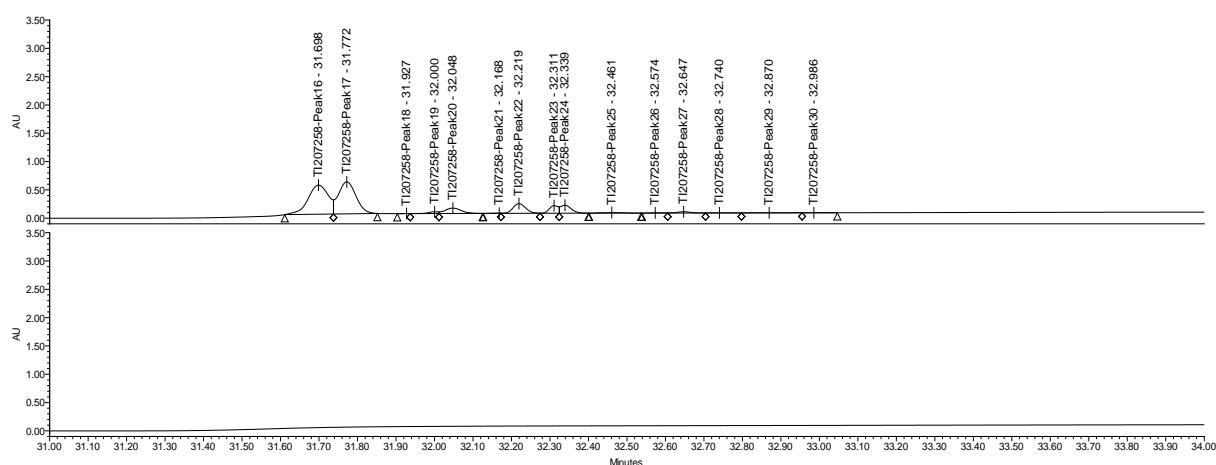


Figure 7
**Zoomed UPLC-UV chromatograms of the 1000 mg/L test solution [top; res. id. 1584]
and corresponding blank [bottom; res. id. 1583], 31-34 minutes**

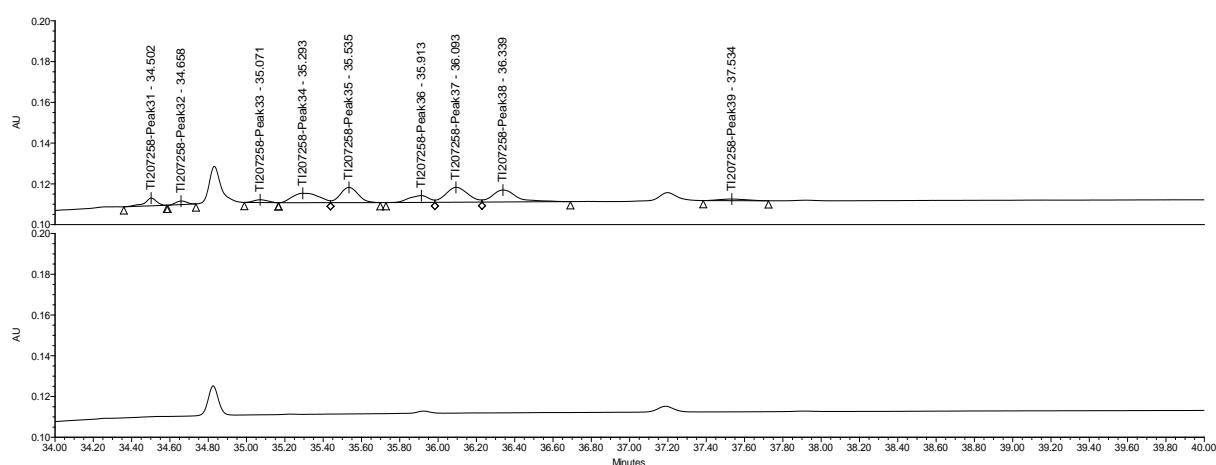


Figure 8
**Zoomed UPLC-UV chromatograms of the 1000 mg/L test solution [top; res. id. 1584]
and corresponding blank [bottom; res. id. 1583], 34-40 minutes**

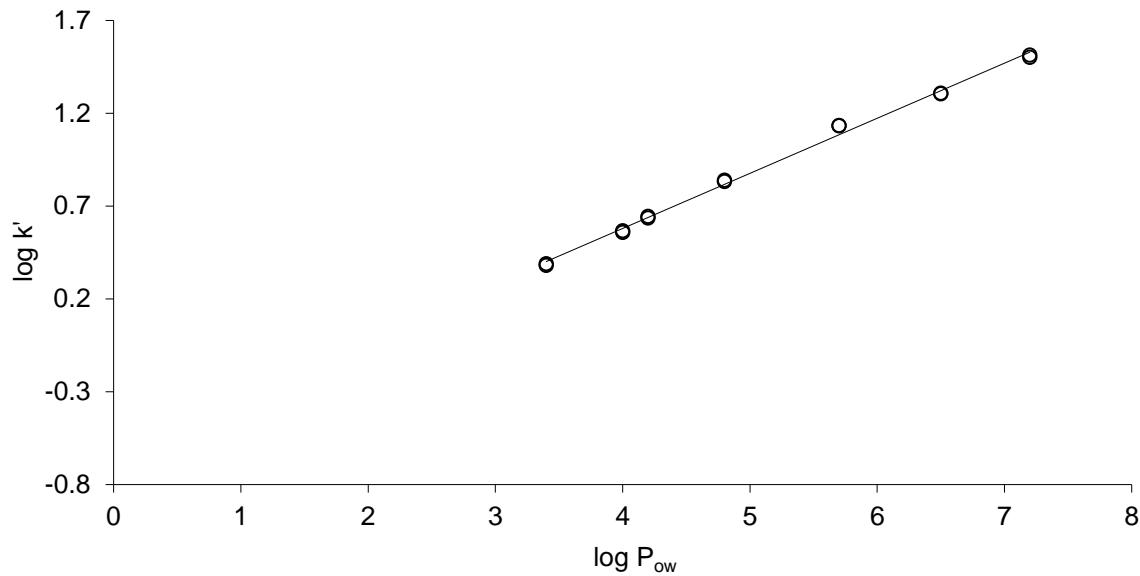


Figure 9
The regression line of the reference substances: $\log k'$ versus $\log P_{ow}$

Appendix 1 Certificate of Analysis

Chemtura Corporation
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C ₂₄ H ₄₇ NO ₃	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C ₂₂ H ₄₅ NO ₃	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C ₂₄ H ₄₅ NO ₃	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C ₂₄ H ₄₉ NO ₃	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C ₂₂ H ₄₃ NO ₃	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C ₂₆ H ₄₅ NO ₃	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C ₂₄ H ₄₃ NO ₃	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C ₁₈ H ₃₄ O ₂	C18:1 (oleic) acid
1.1	421.4	C ₂₆ H ₄₇ NO ₃	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis 3/7/16
 Blake Lewis
 Analytical REACh Scientist, Analytical Services
Colin Moore 3/7/16
 Albert J. Nitowski
 Sr. Technology Manager
 Analytical and Lab Support Services